Bioactive Pentacyclic Triterpenoids from the Leaves of *Cleistocalyx operculatus*

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ABSTRACT. Thirteen new pentacyclic triterpenoids, cleistocalyxic acids A–K (**1**, **2**, **4**, **5**, and **7**–**13**) and cleistocalyxolides A (**3**) and B (**6**), and fifteen known analogues (**14**–**28**), based on taraxastane, oleanane, ursane, multiflorane, and lupane skeletons, were isolated from the leaves of *Cleistocalyx operculatus*. The structures of **1**–**13** were elucidated by analysis of their spectroscopic data and ECD/TDDFT computations. Cleistocalyxolide A (**3**), presumed to be derived from the known taraxastane-type compound **14**, has a rare rearranged triterpenoid backbone. Cleistocalyxic acid B (**2**) displayed cytotoxicity against HepG2, NCI-N87, and MCF-7 cancer cell lines with IC_{50} values ranging from 3.2 to 6.5 μM and cleistocalyxic acid D (**5**) was active against HepG2 and NCI-N87 cells with the values around 5.0 μM. The non-cytotoxic cleistocalyxic acid E (**7**) inhibited production of IL-6 by 68.1% and TNF-α by 53.7% in LPS-induced RAW 264.7 macrophages at the concentration of 2 μM.

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Cleistocalyx operculatus (Roxb.) Merr. et Perry, an evergreen tree belonging to the family Myrtaceae, is widely distributed in southern mainland China and other areas of tropical Asia. Its leaves and flower buds have long been used as herbal tea and herbal medicine in the People's Republic of China and Vietnam for the treatment of cold fever, inflammation, and gastrointestinal disorders.[1] Chemical constituents of the flower buds were extensively investigated and chalcones, flavanones, flavones, and oleanane- and ursane-type triterpenoids were isolated with a wide range of biological activities, including cancer cell growth inhibition,[2] cholinesterase inhibition,[3] antioxidation,[4] anti-hyperglycemia,[5] anti-influenza,[6] and anti-inflammation.[7] Chemical investigations on the leaves were recently reported, in which a new taraxastane-type triterpene, two new acetophenones, and a new flavanone were disclosed.[8,9] In a continuing endeavor to discover biologically active compounds from natural sources,[10−14] the constituents of the leaves of *C. operculatus* were investigated and 13 new pentacyclic triterpenoids (**1**–**13**) along with 15 known compounds (**14**–**28**) were obtained, of which **1** and **2** are the first two taraxastane-type triterpenoids having an aldehyde or carboxy C-27 and **3** has a rare rearranged triterpenoid backbone. The isolated triterpenoids were evaluated for the tumor cell toxicity and in vitro anti-inflammatory activity. Herein are reported the isolation, structural elucidation, and biological activities of these compounds.

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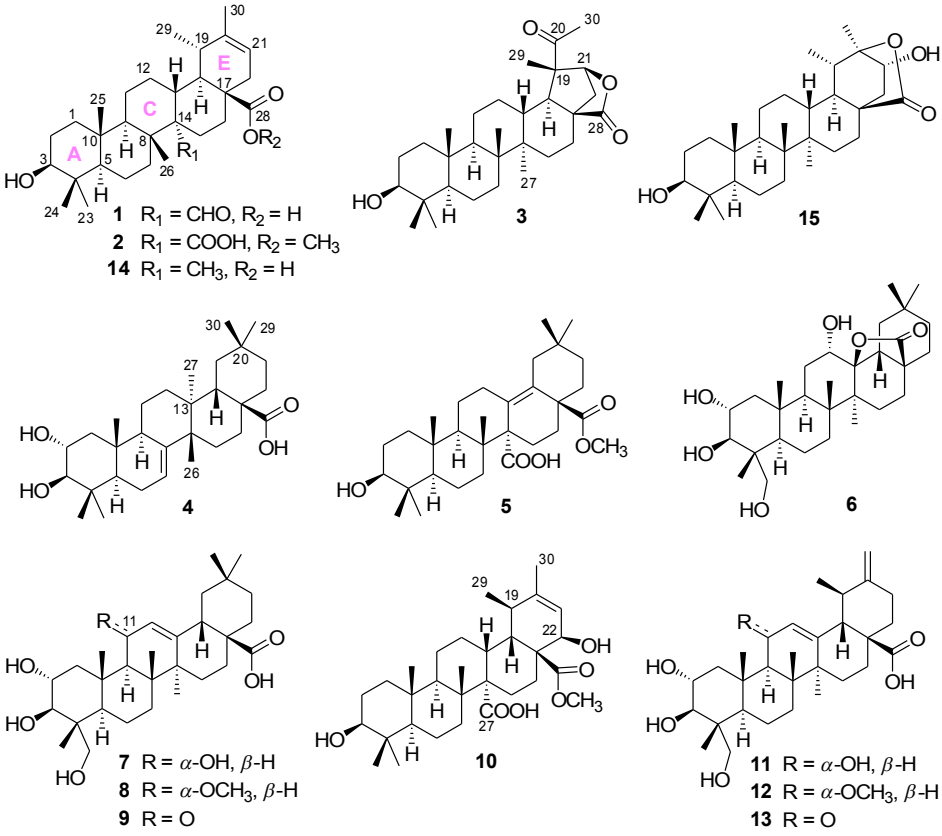

RESULTS AND DISCUSSION

Air dried leaves of *C. operculatus* were powdered and extracted with 90% aqueous EtOH. The crude extract was suspended in water and successively partitioned with petroleum ether, EtOAc, and *n*-BuOH. The combined petroleum ether- and EtOAc-soluble fractions were subjected to a series of column chromatographic purification steps over silica gel, ODS, and Sephadex LH-20, followed by semi-preparative HPLC, to afford 13 new (**1**-**13**) and 15 known (**14**-**28**) pentacyclic triterpenoids, of the oleanane, ursane, taraxastane, multiflorane, and lupane types. The known compounds were identified as 3β-hydroxytaraxast-20-en-28-oic acid (**14**),[15] oleanolic acid (**16**),[16] maslinic acid (**17**),[17] arjunolic acid (**18**),[18] 11α,12α-epoxy-2α,3β,23-trihydroxyolean-28,13β-olide (**19**),[19] 2α-hydroxymicromeric acid (**20**),[20] actinidic acid (**21**),[21] ursolic acid (**22**),[22] corosolic acid (**23**),[23] asiatic acid (**24**),[18] 2α,3β,11α,23-tetrahydroxyurs-12-en-28-oic acid (**25**),[24] 2α,3β-

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dihydroxy-24-nor-urs-4(23),12-dien-28-oic acid (**26**),[25] betulinic acid (**27**),[26] and alphitolic acid (**28**),[27] by comparing their spectroscopic data with literature values. 3β,21α-Dihydroxytaraxast-28,20β-olide (**15**) was reported to be obtained in chemical transformation of 20,21-epoxytaraxstane-3β,28-diol diacetate,[28] but its structure was not confirmed. Herein, it was isolated as a naturally occurring compound for the first time and its structure was assigned unambiguously by detailed analysis of its spectroscopic data. Among these isolated triterpenoids, **17**, **18**, **23**, **24** and the new compound **2** were found to be the most abundant components of the leaves of *C. operculatus*.

Cleistocalyxic acid A (**1**) gave a $[M - H]^-$ ion peak at *m/z* 469.3314 in the negative-ion HRESIMS, appropriate for a molecular formula of $C_{30}H_{46}O_4$. The ^{1}H NMR spectrum (Table 1) displayed resonances for four tertiary methyls (δ_H 0.85, 1.01, 1.07, and 1.16), a secondary methyl [δ_H 1.19 (3H, d, J = 6.6 Hz)], an olefinic methyl (δ_H 1.73), an oxymethine [δ_H 3.39 (1H, dd, J = 10.4, 6.6 Hz)], an olefinic proton [δ_H 5.46 (1H, d, J = 7.0 Hz)], and an aldehyde proton [δ_H 10.49 (1H, s)]. The ^{13}C NMR spectrum (Table 2) showed 30 carbon resonances comprising six methyls, nine methylenes, eight methines, of which one was oxygenated (δ_C 78.2), one was olefinic (δ_C 118.4), and one was an aldehyde (δ_C 211.6), and seven quaternary carbons, including olefinic (δ_C 143.1) and a carboxylic acid (δ_C 178.3) carbons. These 1D NMR data, in combination with the molecular formula, suggested the presence of a pentacyclic triterpenoid acid containing a hydroxy group, a formyl group, and a trisubstituted double bond. Detailed analysis of ^{1}H-^{1}H COSY and HSQC spectra was used to identify structural fragments as shown by the bolded lines in Figure 1. The connectivity of the fragments and functionalities were established by the HMBC spectrum (Figure 1). The long-range correlations of two *gem*-methyl singlets (δ_H 1.01 and 1.16) with C-4, C-5, and the oxymethine carbon (δ_C 78.2) (Figure 1) helped locate the hydroxy group

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at C-3. The remaining two tertiary methyl groups were assigned to C-25 and C-26 by HMBC correlations of their protons (δ_H 0.85 and 1.07) with C-1/C-5/C-9 and C-9/C-14, respectively. The HMBC correlations of H-13 (δ_H 3.14) and H_2-15 with the aldehyde carbon (δ_C 211.6) as well as the correlation of aldehyde proton (δ_H 10.49) with C-13 indicated that the formyl group is attached to C-14. The substitution pattern of ring E was evident on the basis of HMBC correlations from the secondary methyl (δ_H 1.19) to C-18/C-20, from the olefinic methyl (δ_H 1.73) to C-19/C-21, from the olefinic proton (δ_H 5.46) to C-17/C-19, and from H-18 to the carboxylic acid carbon (δ_C 178.3). The relative configuration of **1** was established on the basis of proton coupling constants and NOESY correlations (Figure 1). The large diaxial coupling constant between H-2β and H-3 (J = 10.4 Hz) (Table 1) along with NOE interactions of H-3/H-5, H-3/H_3-23, H-5/H-9, H_3-24/H_3-25, and H_3-25/H_3-26 implied that H-3, H-5, and H-9 are co-facial and α-oriented. In turn, NOE correlations of H_3-26/H-13, and H-13/H-19 suggested H-13 and H-19 are β-oriented, while the absence of any correlation between H_3-26 and H-27 and the presence of correlations of H-27/H-18 and H-18/H_3-29 indicated the α-orientation of the H-18, CHO-27, and CH_3-29 functionalities. These findings also illustrated the *trans*-fusion of the A/B/C/D/E rings, in accordance with compound **1** being a taraxastane-type triterpenoid rather than an ursane-type compound that has the *cis*-fusion of D/E rings and the β-orientation of H-18 and CH_3-29 functionalities. Therefore, the structure of **1** was elucidated as 3β-hydroxytaraxast-20-en-27-al-28-oic acid.

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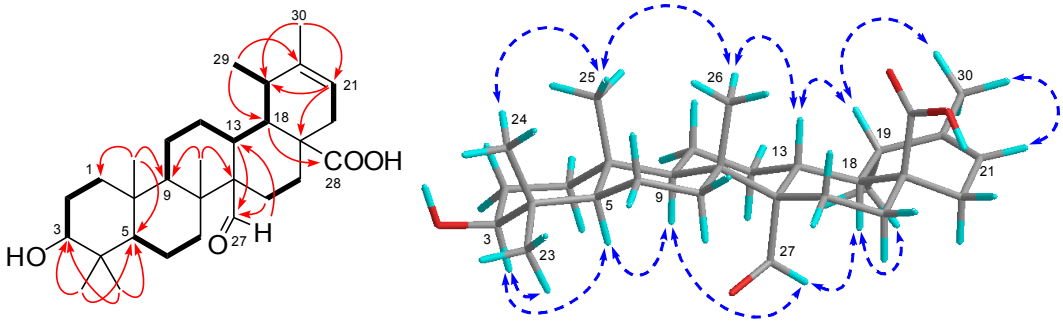

Figure 1. Key ^{1}H-^{1}H COSY (bold lines), HMBC (plain arrows), and NOE (dashed arrows) correlations of **1**.

Cleistocalyxic acid B (**2**) was determined to have a molecular formula of $C_{31}H_{48}O_5$ from the HRESIMS data. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) were almost identical to those of **1** except for the presence of signals for a methoxy group (δ_H 3.68, δ_C 51.7) and replacement of the formyl group in **1** by an additional carboxylic acid carbon (δ_C 176.8). HMBC correlations of H-13 with one carboxylic acid carbon (δ_C 178.5) and H-18/H$_3$-OCH$_3$ with the other one (δ_C 176.8) (Figure S1, Supporting Information) confirmed that **2** possesses a free carboxylic acid group at C-14 and a carboxylic acid methyl ester group at C-18. The relative configuration of **2** was determined to be identical to **1** by interpretation of its NOESY spectrum. Therefore, the structure of **2** was defined as 3β-hydroxytaraxast-20-en-27,28-dioic acid 28-methyl ester. To the best of our knowledge, compounds **1** and **2** represent the first two examples of taraxastane-type triterpenoids having an aldehyde or carboxylic acid substituent at C-27.

Table 1. ^{1}H NMR (500 MHz) Data [δ_H in ppm, mult., (J in Hz)] of Compounds 1–6

no.	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]
1	α 0.94 m β 1.71 m	α 1.05 m β 1.79 m	α 0.99 m β 1.66 m	α 1.46 m β 2.33 dd (12.6, 4.3)	α 1.03 m β 1.75 m	α 0.92 m β 1.99–2.05 m
2	1.83–1.87 m	1.83–1.89 m	1.86–1.92 m	4.13 ddd (11.5, 9.4, 4.3)	1.85–1.92 m 1.77–1.82 m	3.70 ddd (11.5, 9.6, 4.6)
3	3.39 dd (10.4, 6.6)	3.28 dd (11.3, 4.6)	3.48 dd (10.5, 5.8)	3.44 d (9.4)	3.34 dd (11.5, 4.6)	3.37 d (9.6)

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5	0.72 dd (11.9, 1.4)	0.91 dd (12.1, 1.5)	0.82 m	1.50–1.58 m	1.06 m	1.31 m
6	α 1.54 m β 1.34 m	α 1.62 m β 1.41–1.45 m	α 1.57 m β 1.38–1.44 m	α 2.21 m β 2.04 m	α 1.68 m β 1.44 m	1.43–1.48 m
7	1.60–1.68 m	α 2.04 ddd (13.5, 13.0, 3.6) β 1.91–1.96 m	1.38–1.44 m	5.55 m	α 2.77 m β 1.77–1.82 m	α 1.75 m β 1.22 m
9	1.35 m	1.91–1.96 m	1.38–1.44 m	2.41 m	1.94 m	1.77 m
11	α 1.79 m β 1.51 m	α 1.79 m β 1.41–1.45 m	α 1.46 m β 1.27 m	1.67–1.73 m 1.50–1.58 m	α 1.71 m β 1.56 m	α 1.55–1.61 m β 1.93 ddd (13.7, 13.3, 3.8)
12	α 2.26 m β 2.05 m	1.97 m 2.88 m	α 1.25 m β 1.52 m	1.67–1.73 m	α 2.89 m β 3.07 dd (15.3, 5.4)	3.77 dd (3.8, 2.4)
13	3.14 ddd (13.3, 12.7, 3.8)	2.87 m	1.73 m			
15	α 2.13 ddd (13.8, 4.2, 3.0) β 1.60–1.68 m	α 2.38–2.44 m β 1.52 ddd (13.5, 13.0, 3.5)	α 1.14 ddd (14.0, 4.7, 2.6) β 2.60 ddd (14.0, 13.7, 4.3)	1.76 m 1.37 m	α 2.32 m β 1.97 m	α 1.17–1.20 m β 1.81 m
16	α 1.40 ddd (14.0, 13.5, 4.2) β 2.39 ddd (14.0, 4.3, 3.0)	1.91–1.96 m 2.38–2.44 m	α 1.65 m β 2.00 m	α 1.87 m β 1.64 m	α 1.85–1.92 m β 2.13 m	α 2.25 ddd (13.2, 13.0, 5.6) β 1.17–1.20 m
18	1.49 m	1.78 m	2.68 d (12.4)	2.95 dd (11.5, 4.9)		2.07 m
19	2.54 m	2.57 m		1.50–1.58 m 1.42 m	α 2.75 d (13.6) β 1.85–1.92 m	1.99–2.05 m
21	5.46 d (7.0)	5.34 d (6.9)	4.87 d (1.9)	1.99–2.03 m	α 1.26 m β 1.45–1.50 m	α 1.43–1.48 m β 1.27 m
22	α 1.91 m β 2.62 dd (15.5, 7.0)	1.83–1.89 m 2.38–2.44 m	α 1.68 m β 1.96 m	2.58 m 1.85 m	α 1.45–1.50 m β 2.38 m	1.55–1.61 m
23	1.16 s	1.10 s	1.25 s	1.25 s	1.18 s	3.51 d (11.1) 3.27 d (11.1)
24	1.01 s	1.03 s	1.054 s	1.18 s	1.06 s	0.69 s
25	0.85 s	0.96 s	0.86 s	0.89 s	0.98 s	0.99 s
26	1.07 s	1.19 s	1.07 s	1.35 s	1.17 s	1.13 s
27	10.49 s		1.046 s	1.20 s		1.36 s
29	1.19 d (6.6)	1.26 d (6.6)	1.40 s	1.05 s	0.98 s	0.98 s
30	1.73 s	1.70 s	2.29 s	1.25 s	0.97 s	0.93 s
OCH_3		3.68 s			3.78 s	

[a]Pyridine-d_5 used as solvent. [b]Methanol-d_4 used as solvent.

Cleistocalyxolide A (**3**) gave a molecular formula of $C_{30}H_{46}O_4$ as determined from the HRESIMS data. The ^1H and ^{13}C NMR data of **3** (Tables 1 and 2) were closely comparable to those of betulinic acid (**27**)[26] except that the resonances corresponding to the 19-propen-2-yl group, CH-19, and CH_2-20 in **27** were not observed. Instead, the proton and carbon signals for an acetyl group [δ_H 2.29 (s, H_3-30); δ_C 26.6 (C-30), 209.7 (C-20)], an additional tertiary methyl [δ_H

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1.40 (s, H_3-29); δ_C 16.1 (C-29)], an extra aliphatic quaternary carbon [δ_C 59.6 (C-19)], and an oxygenated methine [δ_H 4.87 (d, J = 1.9 Hz, H-21); δ_C 84.3 (C-21)] were observed in the spectra. The presence of H-C correlations of H_3-30 with C-19, H_3-29 with C-20/C-21, H-21 with C-17 (δ_C 52.7)/C-18 (δ_C 45.6)/C-28 (δ_C 178.7), and H-18 (δ_H 2.68) with C-20/C-28 in the HMBC spectrum (Figure 2), together with the spin system of H-21/H_2-22 in the ^{1}H-^{1}H COSY spectrum (Figure 2), revealed the occurrence of a five-membered ring consisting of C-17, C-18, C-19, C-21, and C-22, in which CH_3-29 and an acetyl group attached to C-19 and C-17 carboxylic acid substituent is connected to C-21 via an ester linkage to form a lactone ring. In the NOESY spectrum, NOE correlations were observed for H_3-27/H-18, H-18/H_3-30, H_3-30/H-21, and H-13/H_3-29 (Figure 2), indicating the β-orientation of CH_3-29 and the α-orientation of 19-acetyl and H-21 functionalities. In order to determine the configuration, the possible stereoisomers, (18*S*,19*S*)- and (18*R*,19*R*)-**3**, were subjected to quantum chemical computations of their ECD spectra (see Supporting Information). As can be seen in Figure 3, the calculated ECD spectrum of the (18*S*,19*S*)-isomer, which displayed a negative Cotton effect (CE) at 295 nm and a positive CE at 220 nm, arising from n $\rightarrow$ π^* excitations of keto carbonyl and lactone carboxy functions, matches up well with the measured spectrum. In turn, the predicted ECD spectrum of the (18*R*,19*R*)-isomer, showing the absence of a CE around 295 nm, was inconsistent with the measured ECD data. Accordingly, the structure of **3** was determined as 3β-hydroxy-20-oxo-21(20$\rightarrow$19)-*abeo*-taraxast-28,21β-olide.

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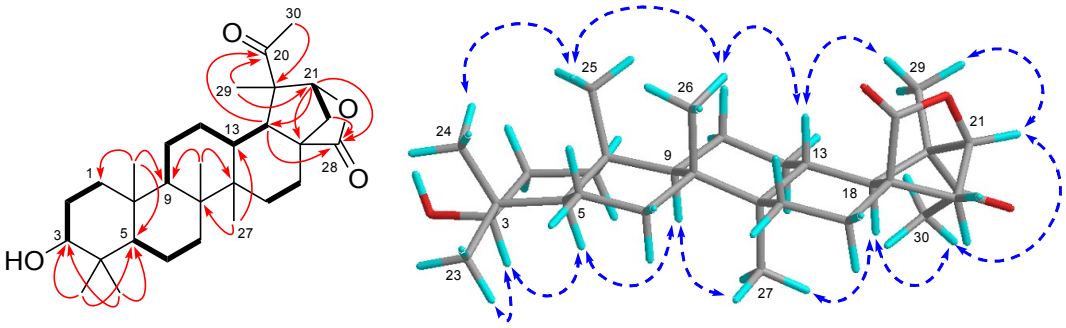

Figure 2. Key ^{1}H-^{1}H COSY (bold lines), HMBC (plain arrows), and NOE (dashed arrows) correlations of **3**.

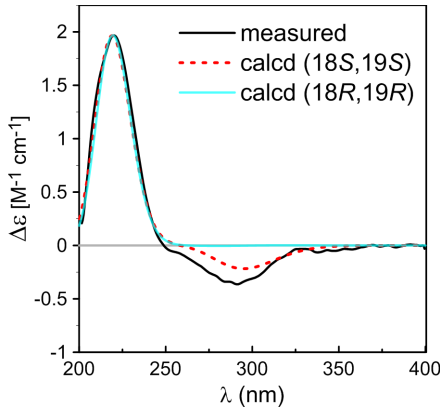

Figure 3. Comparison of the measured ECD spectrum of **3** with M06-2X/TZVP calculated ECD spectra of (18*S*,19*S*)- and (18*R*,19*R*)-**3** in MeOH (σ = 0.35 eV, shift = –6 nm for the (18*S*,19*S*)-isomer and –2 nm for the (18*R*,19*R*)-isomer).

Table 2. ^{13}C NMR (125 MHz) Data (δ_C in ppm, type) of Compounds 1–6

no.	**1**[a]	**2**[a]	**3**[a]	**4**[a]	**5**[a]	**6**[b]
1	39.3, CH_2	39.7, CH_2	39.7, CH_2	46.9, CH_2	39.6, CH_2	48.0, CH_2
2	28.6, CH_2	28.8, CH_2	28.8, CH_2	69.0, CH	28.7, CH_2	69.9, CH
3	78.2, CH	78.3, CH	78.5, CH	84.1, CH	78.4, CH	78.1, CH
4	39.9, C	39.9, C	40.0, C	40.3, C	39.9, C	44.2, C
5	56.0, CH	56.5, CH	56.2, CH	51.1, CH	55.8, CH	48.2, CH
6	19.0, CH_2	19.4, CH_2	19.2, CH_2	25.1, CH_2	19.6, CH_2	18.5, CH_2
7	35.7, CH_2	38.6[c], CH_2	35.0, CH_2	119.2, CH	37.0, CH_2	34.5, CH_2
8	43.4, C	41.6, C	41.8, C	149.0, C	42.8, C	43.5, C
9	54.6, CH	52.5, CH	51.3, CH	50.3, CH	53.2, CH	45.7, CH
10	38.3, C	38.3, C	37.9, C	37.1, C	38.5, C	38.6, C

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11	22.4, CH_2	22.3, CH_2	21.4, CH_2	18.3, CH_2	21.8, CH_2	29.7, CH_2
12	27.6, CH_2	28.3[d], CH_2	27.8, CH_2	37.8, CH_2	27.6, CH_2	76.6, CH
13	39.2, CH	41.2, CH	37.1, CH	37.6, C	135.2, C	93.3, C
14	57.8, C	59.8, C	43.6, C	41.9, C	60.0, C	43.4, C
15	24.8, CH_2	28.4[d], CH_2	27.4, CH_2	34.5[c], CH_2	25.0, CH_2	29.0, CH_2
16	34.6, CH_2	36.2, CH_2	24.5, CH_2	34.8, CH_2	34.7, CH_2	22.3, CH_2
17	49.1, C	49.7, C	52.7, C	46.0, C	49.5, C	46.2, C
18	51.0, CH	51.9, CH	45.6, CH	42.1, CH	131.2, C	52.5, CH
19	38.5, CH	38.0, CH	59.6, C	36.3, CH_2	42.4, CH_2	40.2, CH_2
20	143.1, C	143.5, C	209.7, C	29.5, C	34.2, C	32.4, C
21	118.4, CH	118.1, CH	84.3, CH	34.6[c], CH_2	37.9, CH_2	35.2, CH_2
22	38.6, CH_2	38.7[c], CH_2	44.8, CH_2	31.6, CH_2	36.4, CH_2	28.7, CH_2
23	28.9, CH_3	29.0, CH_3	29.1, CH_3	29.4, CH_3	29.2, CH_3	66.1, CH_2
24	16.8, CH_3	17.0, CH_3	16.5, CH_3	17.3, CH_3	17.13[c], CH_3	13.7, CH_3
25	17.1, CH_3	17.6, CH_3	16.8[c], CH_3	15.0, CH_3	17.10[c], CH_3	18.4, CH_3
26	17.6, CH_3	18.0, CH_3	16.9[c], CH_3	26.3, CH_3	19.2, CH_3	19.2, CH_3
27	211.6, CH	178.5, C	15.1, CH_3	29.6, CH_3	179.0, C	19.0, CH_3
28	178.3, C	176.8, C	178.7, C	181.7, C	177.5, C	182.6, C
29	23.9, CH_3	23.6, CH_3	16.1, CH_3	34.1, CH_3	32.8, CH_3	33.7, CH_3
30	22.6, CH_3	22.5, CH_3	26.6, CH_3	31.8, CH_3	24.8, CH_3	24.2, CH_3
OCH_3		51.7, CH_3			52.3, CH_3	

[a]Pyridine-d_5 used as solvent. [b]Methanol-d_4 used as solvent. [c,d]Assignments interchangeable in the same column.

The triterpenoid backbone represented by **3** is not seen commonly in Nature, for which only two compounds of this kind were previously reported.[29, 30] Instead of being derived from a lupane skeleton,[29] **3** is likely generated from the co-occurring known compound 3β-hydroxytaraxast-20-en-28-oic acid (**14**) by oxidative cleavage of the double bond, subsequent aldol condensation, and formation of the final lactone ring (Scheme 1).

Scheme 1. Possible biogenetic pathway of **3** from **14**.

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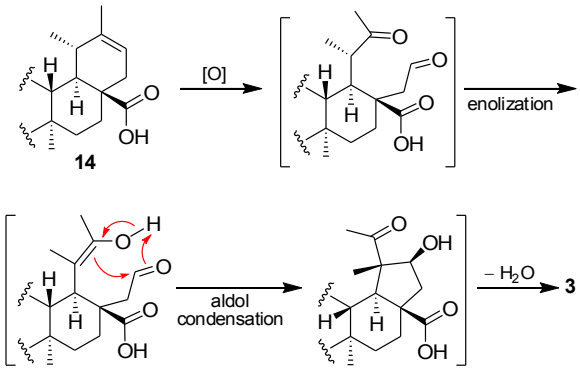

The molecular formula of cleistocalyxic acid C (**4**) was found to be $C_{30}H_{48}O_4$ on the basis of the HRESIMS data. The ^1H and ^{13}C NMR spectra (Tables 1 and 2), in combination with the HSQC spectrum, indicated that **4** is a pentacyclic triterpenoid with seven tertiary methyls, two oxygen-bearing methines [δ_H 4.13 (ddd, J = 11.5, 9.4, 4.3 Hz, H-2), 3.44 (d, J = 9.4 Hz, H-3); δ_C 69.0 (C-2), 84.1 (C-3)], a trisubstituted double bond [δ_H 5.55 (m, H-7); δ_C 119.2 (C-7), 149.0 (C-8)], and a carboxylic acid group [δ_C 181.7 (C-28)]. Analysis of the ^1H-^1H COSY and HMBC spectra was used to establish a multiflorane type triterpenoid,[31, 32] in which hydroxylation at C-2 and C-3 was determined by the HMBC correlations of two *gem*-methyl singlets (δ_H 1.18 and 1.25) with C-3 and C-5, and the strong ^1H-^1H COSY interaction between H-2 and H-3 (Figure 4). The presence of a double bond between C-7 and C-8 was deduced from the ^1H-^1H COSY spin system of H-5/H_2-6/H-7 along with HMBC correlations of H_2-6/H_3-26 with C-8 and of H-7 with C-9/C-14 (Figure 4). Also, the carboxylic acid group was assigned to C-28 by the HMBC cross peaks observed from H_2-15/H-18/H_2-22 to the carboxy carbon (δ_C 181.7). The relative configuration of **4** was established on the basis of proton coupling constants and NOESY spectrum. The large diaxial coupling constants between H-1α and H-2 (J = 11.5 Hz), and between H-2 and H-3 (J = 9.4 Hz) (Table 1) as well as NOE interactions of H-3/H-5, H-3/H_3-23, H-5/H-9, and H-9/H_3-27 (Figure 4) indicated that H-3, H-5, H-9, CH_3-27 are co-facial and α-oriented. In turn, NOE

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correlations of H-2/H_3-24, H-2/H_3-25, H_3-24/H_3-25, and H_3-26/H-18, together with the absence of any NOE interaction between H-18 and H_3-27 (Figure 4), suggested the β-orientation of H-2 and H-18. Thus, the structure of **4** was proposed as 2α,3β-dihydroxymultiflor-7-en-28-oic acid.

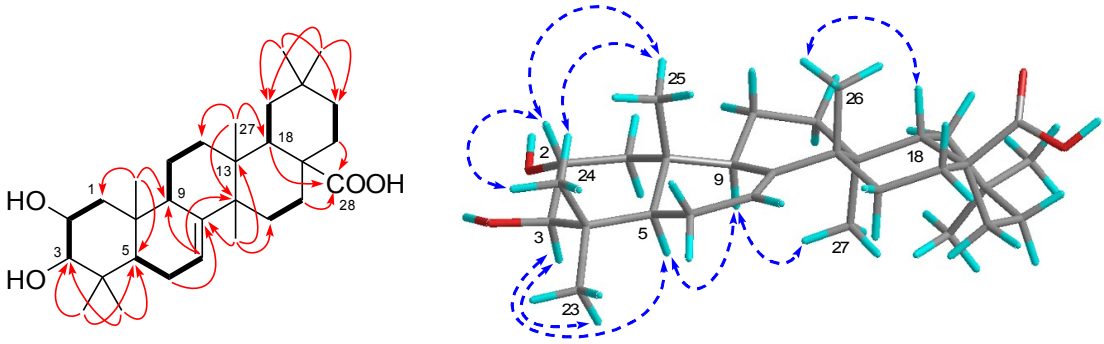

Figure 4. Key ^{1}H-^{1}H COSY (bold lines), HMBC (plain arrows), and NOE (dashed arrows) correlations of **4.**

Cleistocalyxic acid D (**5**) was determined to have the molecular formula, $C_{31}H_{48}O_5$, from the HRESIMS. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) showed a structure closely related to oleanolic acid (**16**) except for the presence of a methoxy group (δ_H 3.78; δ_C 52.3) and replacements of the trisubstituted olefin and one of the tertiary methyls in **16** by a tetrasubstituted double bond (δ_C 135.2 and 131.2) and a carboxylic acid group (δ_C 177.5 or 179.0), respectively. Analysis of the ^{1}H-^{1}H COSY and HSQC spectra (Figure S1, Supporting Information) enabled the assignment of a methylene (δ_H 3.07, 2.89; δ_C 27.6) to C-12, but showed the absence of a methine at C-18 as in compound **16**. Furthermore, the HMBC spectrum (Figure S1, Supporting Information) showed long-range H-C correlations from H_2-12 and H_2-19 to both olefinic carbons, revealing that a double bond is located between C-13 and C-18. Carboxylic acid groups at C-14 and C-17, with the latter being esterified by a methyl group, were shown by HMBC correlations from H_2-15 to one carboxy carbon (δ_C 179.0) and from H_2-16, H_2-22, and the methoxy protons to

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the other carboxy carbon (δ_C 177.5) (Figure S1, Supporting Information). The relative configuration of **5** was found to be consistent with that of **16** by the proton coupling constants (Table 1) and NOE interactions in the NOESY spectrum (Supporting Information). Therefore, the structure of **5** was elucidated as 3β-hydroxyolean-13(18)-en-27,28-dioic acid 28-methyl ester.

Cleistocalyxolide B (**6**) showed a molecular formula of $C_{30}H_{48}O_6$, according to the HRESIMS data. The ^{1}H and ^{13}C NMR spectra of **6** (Tables 1 and 2) were very similar to those of 2α,3β,23-trihydroxy-11α,12α-epoxyolean-28,13β-olide (**19**) except for the absence of the resonances for a 11,12-epoxide ring. Instead, the spectra exhibited signals for an extra hydroxylated methine (δ_H 3.77; δ_C 76.6) and an additional methylene (δ_H 1.93, 1.58; δ_C 29.7), which were assigned to C-12 and C-11, respectively, by analysis of ^{1}H-^{1}H COSY (Figure 5) and HSQC spectra. The assignments were supported by HMBC correlations of H_2-11 with C-12 and C-13 (δ_C 93.3), and of H_3-27 with C-13 (Figure 5). The α-orientation of OH-12 was suggested by the NOE correlation of H-12/H-18 in the NOESY spectrum (Figure 5) and the small ^{1}H NMR coupling constants between H-12 and H_2-11 (J = 3.8 and 2.4 Hz). The stereochemistry of **6** was supported by theoretical ECD computations (Supporting Information), which were consistent with the negative CE around 220 nm in the measured ECD spectrum (Figure 6). Accordingly, the structure of **6** was characterized as 2α,3β,12α,23-tetrahydroxyolean-28,13β-olide.

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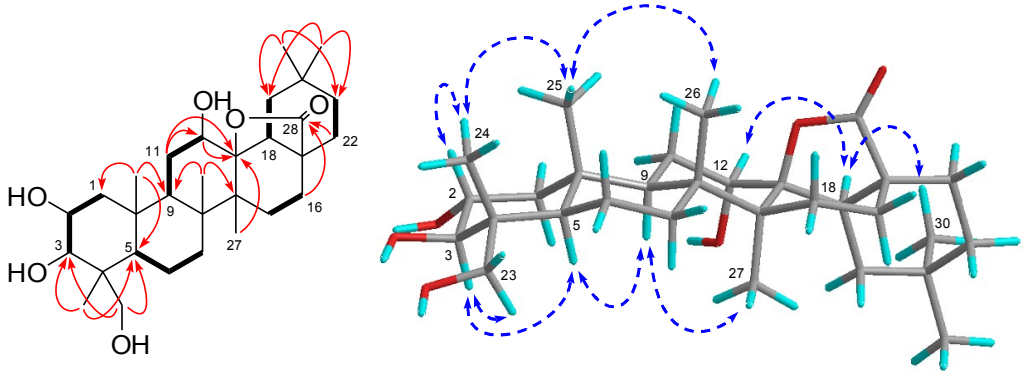

Figure 5. Key ^{1}H-^{1}H COSY (bold lines), HMBC (plain arrows), and NOE (dashed arrows) correlations of **6**.

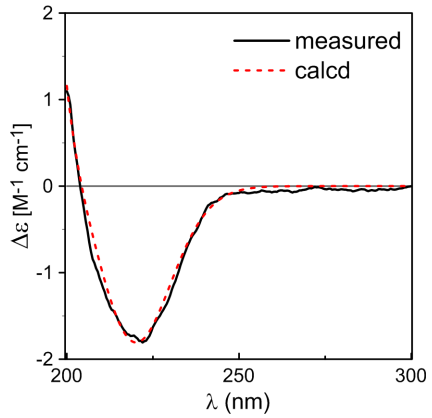

Figure 6. Comparison between the M06/TZVP calculated and measured ECD spectra of **6** in MeOH (σ = 0.36 eV, shift = +1 nm).

Cleistocalyxic acid E (**7**) gave a sodium adduct ion peak at *m/z* 527.3325 in (+)-HRESIMS, corresponding to a molecular formula of $C_{30}H_{48}O_6$. The ^{1}H and ^{13}C NMR spectra (Tables 3 and 4) were almost identical to those of arjunolic acid (**18**) except for replacement one of the methylenes in **18** by an additional oxymethine [δ_H 4.44 (1H, dd, J = 8.8, 3.8 Hz); δ_C 81.9] in **7**. The ^{1}H-^{1}H COSY interactions of this oxymethine proton with H-9 and H-12 as well as HMBC correlations of the same oxymethine proton with C-10/C-12/C-13 (Figure 7) revealed the attachment of an OH group to C-11 in **7**. The NOE interation of H_3-26 with H-11 (Figure 7)

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indicated the α-orientation of OH-11. Cleistocalyxic acid F (**8**) gave a molecular formula with one CH_2 unit more than that of **7** according to its (+)-HRESIMS. It was an 11-*O*-methylated derivative of **7** as indicated by close similarity of its ^{1}H and ^{13}C NMR spectra with those of **7**, except for the presence of signals for a methoxy group (δ_H 3.29; δ_C 54.6) and the upfield shifts of H-11 and C-11 relative to those in **7** (Tables 3 and 4), and supported by its 2D NMR spectra (Figure S1, Supporting Information). Therefore, the structures of **7** and **8** were defined as 2α,3β,11α,23-tetrahydroxyolean-12-en-28-oic acid and 2α,3β,23-trihydroxy-11α-methoxyolean-12-en-28-oic acid, respectively.

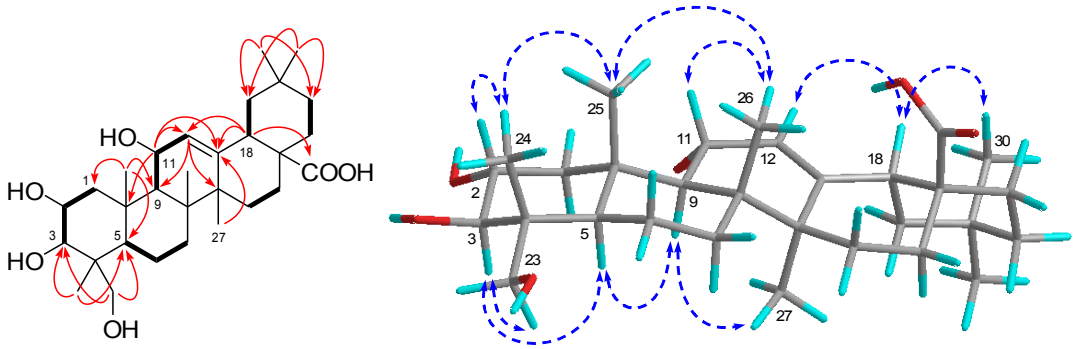

Figure 7. Key ^{1}H-^{1}H COSY (bold lines), HMBC (plain arrows), and NOE (dashed arrows) correlations of **7**.

Table 3. ^{1}H NMR Data (500 MHz) [δ_H in ppm, mult., (*J* in Hz)] of Compounds 7–13

no.	**7**[b]	**8**[b]	**9**[b]	**10**[a]	**11**[b]	**12**[b]	**13**[b]
1	α 1.15 m β 2.33 dd (13.3, 4.4)	α 1.14–1.20 m β 2.17 dd (13.3, 4.4)	α 0.92 m β 3.08 dd (12.7, 4.4)	α 1.07 m β 1.79 m	α 1.11–1.17 m β 2.45 dd (13.3, 4.3)	α 1.19 m β 2.20 dd (13.7, 5.2)	α 0.93 m β 3.05 dd (12.7, 4.5)
2	3.70 ddd (11.8, 9.6, 4.4)	3.69 ddd (11.6, 9.6, 4.3)	3.74 ddd (11.5, 9.6, 4.5)	1.81–1.86 m 1.88 m	3.70 ddd (11.9, 9.7, 4.4)	3.70 ddd (11.5, 9.6, 4.3)	3.75 ddd (11.3, 9.7, 4.4)
3	3.36 d (9.6)	3.35 d (9.6)	3.35 d (9.6)	3.28 dd (11.5, 4.6)	3.37 d (9.7)	3.36 d (9.6)	3.37 d (9.7)
5	1.30 m	1.31 m	1.24–1.28 m	0.91 m	1.32 m	1.30 m	1.27 m
6	α 1.49 m β 1.38–1.44 m	α 1.48 m β 1.38 m	α 1.50 m β 1.44 m	α 1.62 m β 1.45 m	α 1.49 m β 1.41 m	α 1.49 m β 1.40 m	α 1.52 m β 1.44 m
7	1.54–1.59 m 1.20–1.24 m	1.54–1.59 m 1.19–1.24 m	1.74–1.80 m 1.33 m	α 2.07 m β 1.99 m	α 1.62 m β 1.27 m	α 1.61 m β 1.25 m	α 1.81 m β 1.38 m

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9	1.78 d (8.8)	1.80 d (8.6)	2.52 s	1.97 m	1.84 d (9.2)	1.79 d (8.5)	2.52 s
11	4.44 dd (8.8, 3.8)	3.88 dd (8.6, 3.8)		α 1.81–1.86 m β 1.50 m	4.47 dd (9.2, 3.4)	3.85 dd (8.5, 3.6)	
12	5.60 d (3.8)	5.62 dd (3.8)	5.56 s	α 2.97 m β 2.05 m	5.57 d (3.4)	5.51 d (3.6)	5.55 s
13				2.99 m			
15	α 1.09 m β 1.71 m	α 1.09 m β 1.70 m	α 1.29 m β 1.74–1.80 m	α 2.52 ddd (13.4, 4.6, 3.3) β 1.59 m	α 1.11–1.17 m β 1.89 m	α 1.16 m β 1.85–1.90 m	α 1.33 m β 1.92–1.98 m
16	α 2.04 ddd (14.4, 13.4, 3.8) β 1.62 m	α 2.04 ddd (14.4, 13.5, 4.1) β 1.63 m	α 2.14 ddd (14.1, 13.4, 4.7) β 1.72 m	α 2.88 ddd (14.0, 13.6, 3.3) β 2.39 ddd (14.0, 4.6, 3.2)	α 2.25 m β 1.77 m	α 2.28 m β 1.77 m	α 2.35 m β 1.86 m
18	2.92 dd (13.8, 4.6)	2.90 dd (14.0, 4.6)	3.00 dd (14.1, 4.4)	2.62 m	2.32–2.36 m	2.31 m	2.46 m
19	α 1.65 m β 1.33 m	α 1.68 m β 1.14–1.20 m	α 1.74–1.80 m β 1.19 m	2.64 m	2.37 m	2.40 m	2.49 m
21	1.38–1.44 m 1.20–1.24 m	1.41 m 1.19–1.24 m	1.47 m 1.24–1.28 m	5.89 d (6.5)	2.32–2.36 m 2.22 m	2.36 m 2.24 m	1.92–1.98 m 1.64 m
22	α 1.54–1.59 m β 1.76 m	α 1.54–1.59 m β 1.75 m	α 1.66 m β 1.74–1.80 m	4.53 d (6.5)	1.86 m 1.64 m	1.85–1.90 m 1.64 m	2.39 m 2.25 m
23	3.51 d (11.2) 3.27 d (11.2)	3.51 d (11.2) 3.26 d (11.2)	3.52 d (11.2) 3.26 d (11.2)	1.10 s	3.52 d (11.2) 3.27 d (11.2)	3.52 (11.1) 3.27 (11.1)	3.53 d (11.2) 3.26 d (11.2)
24	0.70 s	0.70 s	0.70 s	1.04 s	0.71 s	0.71 s	0.70 s
25	1.13 s	1.13 s	1.20 s	0.97 s	1.16 s	1.15 s	1.22 s
26	0.84 s	0.83 s	0.98 s	1.24 s	0.89 s	0.85 s	1.02 s
27	1.24 s	1.25 s	1.43 s		1.25 s	1.26 s	1.43 s
29	0.92 s	0.93 s	0.95 s	1.36 d (6.0)	1.14 d (5.9)	1.09 d (6.4)	1.03 d (6.0)
30	0.97 s	0.97 s	0.97 s	1.78 s	4.70 brs 4.66 brs	4.71 brs 4.67 brs	4.75 brs 4.69 brs
OCH_3		3.29 s		3.71 s		3.33 s	

[a]Pyridine-d_5 used as solvent. [b]Methanol-d_4 used as solvent.

Cleistocalyxic acid G (**9**) was determined to have a molecular formula of $C_{30}H_{46}O_6$ from the HRESIMS, which was two hydrogen atoms less than that of **7**. The 1D NMR data of **9** (Tables 3 and 4) were closely comparable to those of **7** except for the absence of the oxymethine C-11, the presence of a ketone carbonyl carbon (δ_C 202.7), and the significant downfield shifts of C-13 (δ_C 172.8), H-9 (δ_H 2.52), and C-9 (δ_C 62.9) (Tables 3 and 4). Analysis of the 2D NMR spectra (Figure S1, Supporting Information) indicated that **9** is an 11-keto derivative of **7**. The configuration determination of **9** was supported by ECD/TDDFT computations that afforded a

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simulated ECD spectrum in excellent agreement with the measured spectrum (Figure 8). Consequently, the structure of this molecule was established as 2α,3β,23-trihydroxy-11-oxo-olean-12-en-28-oic acid.

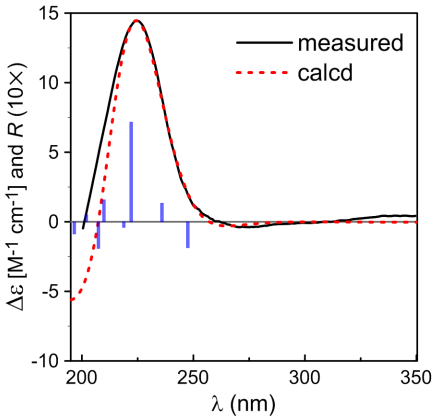

Figure 8. Comparison between the M06/TZVP calculated and measured ECD spectra of **9** in MeOH. Bars represent the calculated rotatory strengths R (10^{-40} cgs) of the global energy minimum (σ = 0.38 eV, shift = –2 nm).

Cleistocalyxic acid H (**10**) was found to have a molecular formula of $C_{31}H_{48}O_6$ on the basis of the HRESIMS. Comprehensive analysis of ^{1}H, ^{13}C, ^{1}H-^{1}H COSY, HSQC, and HMBC NMR spectra (Tables 3 and 4, Figure 9) were used to construct a planar structure similar to those of **2** except for the replacement of the CH_2-22 in **2** by a hydroxymethine [δ_H 4.53, (1H, d, J = 6.5 Hz); δ_C 69.4]. However, the relative configuration of **10** was found to be different from that of **2** on the basis of the NOESY spectrum, in which the presence of NOE correlations of H-12β/H-18, H-16α/H-19, and H-22/H-16β (Figure 9) suggested the β-orientation of H-18, CH_3-29, and OH-22 and an ursane-type *cis* D/E ring junction rather than the taraxastane-type *trans*-fusion of D/E rings in **2**. Hence, the structure of **10** was characterized as 3β,22β-dihydroxyurs-20-en-27,28-dioic acid 28-methyl ester.

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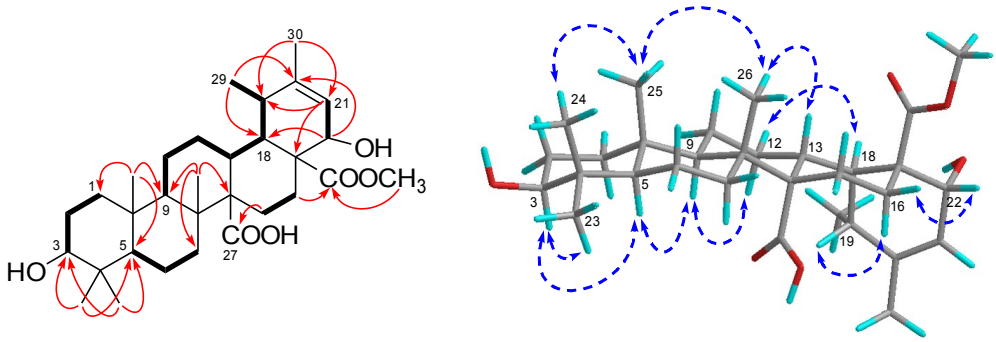

Figure 9. Key ^{1}H-^{1}H COSY (bold lines), HMBC (plain arrows), and NOE (dashed arrows) correlations of **10**.

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Table 4. ^{13}C NMR (125 MHz) Data (δ_C in ppm, type) of Compounds 7–13

no.	**7**[b]	**8**[b]	**9**[b]	**10**[a]	**11**[b]	**12**[b]	**13**[b]
1	48.9, CH_2	48.9, CH_2	48.2, CH_2	39.7, CH_2	49.1, CH_2	49.1, CH_2	48.2, CH_2
2	69.8, CH	69.8, CH	69.3, CH	28.6, CH_2	69.9, CH	69.8, CH	69.4, CH
3	77.9, CH	77.8, CH	77.6, CH	78.3, CH	77.9, CH	77.9, CH	77.7, CH
4	44.2, C	44.1, C	44.2, C	39.9, C	44.2, C	44.2, C	44.2, C
5	48.1, CH	48.2, CH	47.8, CH	56.4, CH	48.2, CH	48.2, CH	47.8, CH
6	19.1, CH_2	19.1, CH_2	18.1, CH_2	19.4, CH_2	19.0, CH_2	19.1, CH_2	18.2, CH_2
7	33.6[c], CH_2	33.6[c], CH_2	33.4, CH_2	38.7, CH_2	34.0, CH_2	33.9, CH_2	33.6, CH_2
8	43.9, C	43.9, C	45.4, C	41.6, C	43.8, C	43.7, C	46.0, C
9	52.2, CH	53.8, CH	62.9, CH	52.5, CH	51.3, CH	54.1, CH	62.6, CH
10	39.8, C	40.1, C	39.1, C	38.3, C	39.9, C	40.0, C	39.1, C
11	81.9, CH	77.4, CH	202.7, C	22.5, CH_2	82.1, CH	77.7, CH	202.2, C
12	123.7, CH	122.6, CH	128.2, CH	28.8, CH_2	127.5, CH	126.0, CH	131.1, CH
13	150.6, C	150.5, C	172.8, C	40.9, CH	144.2, C	143.9, C	166.3, C
14	43.1, C	43.2, C	45.1, C	59.8, C	43.4, C	43.5, C	45.5, C
15	29.0, CH_2	29.0, CH_2	28.9, CH_2	28.4, CH_2	29.4, CH_2	29.3, CH_2	29.6, CH_2
16	23.9, CH_2	23.9, CH_2	23.9, CH_2	32.0, CH_2	25.3, CH_2	25.2, CH_2	25.2, CH_2
17	47.2, C	47.3, C	47.2, C	54.9, C	49.3, C	48.8, C	48.2, C
18	42.2, CH	42.4, CH	43.4, CH	42.8, CH	55.9, CH	55.9, CH	56.7, CH
19	46.4, CH_2	47.0, CH_2	45.5, CH_2	38.3, CH	38.5, CH	38.2, CH	37.8, CH
20	31.6, C	31.6, C	31.6, C	147.5, C	154.6, C	154.2, C	153.4, C
21	34.9, CH_2	34.8, CH_2	34.7, CH_2	123.1, CH	33.3, CH_2	33.2, CH_2	39.9, CH_2
22	33.7[c], CH_2	33.8[c], CH_2	32.9, CH_2	69.4, CH	40.5, CH_2	40.3, CH_2	33.1, CH_2
23	66.1, CH_2	66.1, CH_2	65.8, CH_2	29.0, CH_3	66.2, CH_2	66.2, CH_2	65.9, CH_2
24	13.8, CH_3	13.8, CH_3	13.9, CH_3	17.0, CH_3	13.9, CH_3	13.9, CH_3	13.9, CH_3

25	18.9, CH_3	19.0, CH_3	18.3, CH_3	17.6, CH_3	18.8, CH_3	19.1, CH_3	18.4, CH_3
26	19.6, CH_3	19.4, CH_3	19.8, CH_3	18.1, CH_3	19.6, CH_3	19.4, CH_3	19.9, CH_3
27	25.3, CH_3	25.7, CH_3	24.0, CH_3	178.6, C	23.0, CH_3	23.3, CH_3	21.3, CH_3
28	182.0, C	181.7, C	180.8, C	177.0, C	n.d.[d]	180.7, C	n.d.[d]
29	33.5, CH_3	33.5, CH_3	33.2, CH_3	23.5, CH_3	16.9, CH_3	16.8, CH_3	16.8, CH_3
30	24.0, CH_3	24.0, CH_3	23.8, CH_3	22.8, CH_3	105.4, CH_2	105.6, CH_2	106.2, CH_2
OCH_3		54.6, CH_3		51.7, CH_3		55.1, CH_3	

[a]Pyridine-d_5 used as solvent. [b]Methanol-d_4 used as solvent. [c]Assignments interchangeable in the same column. [d]n.d.: not detected.

Cleistocalyxic acid I (**11**) gave a molecular formula of $C_{30}H_{46}O_6$ as determined from HRESIMS data. The ^1H and ^{13}C NMR spectra (Tables 3 and 4), in combination with the HSQC spectrum, suggested a structure closely related to that of the known compound 2α,3β,11α,23-tetrahydroxyurs-12-en-28-oic acid (**25**), except for replacement one of the aliphatic methines and one of two secondary methyls in **25** by an extra olefinic quaternary carbon (δ_C 154.6) and an olefinic methylene (δ_H 4.66, 4.70; δ_C 105.4), respectively, in accordance with the presence of an exocyclic double bond. Detailed analysis of the ^1H-^1H COSY and HMBC spectra (Figure S1, Supporting Information), in particular HMBC correlations from the olefinic methylene protons to C-19/C-21 and from H_3-29 to the olefinic quaternary carbon (δ_C 154.6), were used to locate the exocyclic double bond at C-20. Cleistocalyxic acid J (**12**) was deduced to be an 11-*O*-methyl derivative of **11** by comparison of its HRESIMS and 1D NMR data with those of **11** (Tables 3 and 4) and analysis of its 2D NMR data (Figure S1, Supporting Information). Consequently, **11** and **12** were defined structurally as 2α,3β,11α,23-tetrahydroxyurs-12,20(30)-dien-28-oic acid and 2α,3β,23-trihydroxy-11α-methoxyurs-12,20(30)-dien-28-oic acid, respectively.

Cleistocalyxic acid K (**13**) was determined to have a molecular formula of $C_{30}H_{44}O_6$, containing two hydrogen atoms less than that of **11**. The ^1H and ^{13}C NMR spectra (Tables 3 and 4) were similar to those of **11** except for resonances corresponding to the protons and carbons in

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ring C, which, in turn, were similar to those of **9**, suggesting this compound to be an 11-keto derivative of **11**. The structure proposed was supported by the 2D NMR data (Figure S1, Supporting Information). The configuration of **13** was substantiated by ECD/TDDFT computations which provided a calculated ECD spectrum consistent with the experimental spectrum (Figure 10). Therefore, **13** was assigned as 2α,3β,23-trihydroxy-11-oxours-12,20(30)-dien-28-oic acid.

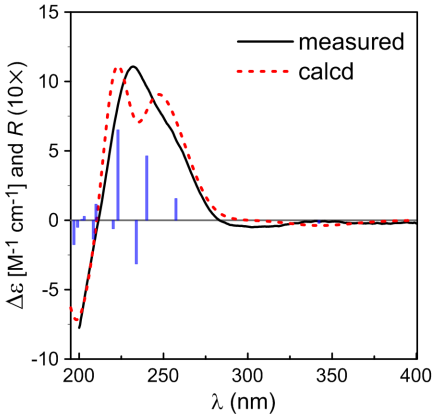

Figure 10. Comparison between the M06/TZVP calculated and measured ECD spectra of **13** in MeOH. Bars represent the calculated rotatory strengths R (10^{-40} cgs) of the global energy minimum (σ = 0.28 eV, shift = –2 nm).

The molecular formula of compound **15** was determined to be $C_{30}H_{48}O_4$ from the HRESIMS. Comparison of ^{1}H and ^{13}C NMR data obtained for **15** and **14** indicated that these two compounds share the same structure in rings A, B, C and D, but have a different ring E. The absence of signals for an olefin moiety and the presence of resonances for an oxygenated quaternary carbon (δ_C 85.4) and an additional oxymethine [δ_H 4.14 (1H, dd, J = 10.2, 5.7); δ_C 72.1] in the NMR spectra of **15** (Experimental Section) suggested that C-20 and C-21 are oxygenated sp^3 carbons in **15** instead of having a double bond between them in **14**. The presence of a weak correlation of

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H_3-30 with the carboxy C-28 (δ_C 176.9) in the HMBC spectrum (Figure S1, Supporting Information), the downfield shift of the oxygenated quaternary carbon C-20, and the upfield shift of C-28, together with the requirement of unsaturation sites indicated substitution of an OH at C-21 and the connection of C-20 to C-28 via a ester linkage to form a six-membered lactone ring. The absence of the NOE correlation between H_3-29 and H-21 (Figure S2, Supporting Information) and the proton coupling pattern of H-21 (dd, J = 10.2, 5.7 Hz) were consistent with a boat conformation of ring E and an α-orientation of OH-21 were also inferred. To determine the configuration of **15**, ECD/TDDFT computations were carried out on two possible stereoisomers, (18*S*,19*S*)- and (18*R*,19*R*)-**15**, representing taraxastane- and ursane-type structures, respectively. As a result (Figure 11), the theoretical ECD spectrum of (18*S*,19*S*)-**15** was found to be almost superimposable on the measured spectrum while that of (18*R*,19*R*)-**15** was similar to the mirror image of the measured one. Consequently, the structure of this molecule was defined as 3β,21α-dihydroxytaraxast-28,20β-olide.

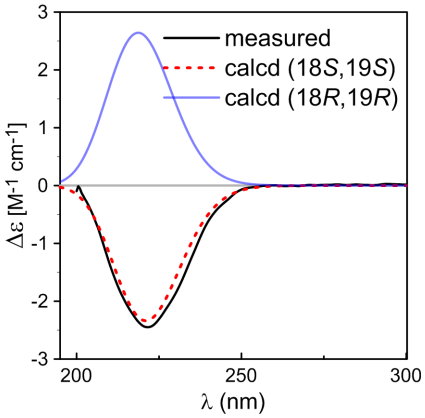

Figure 11. Comparison of the measured ECD spectrum of **15** with M06-2X/TZVP calculated spectra of (18*S*,19*S*)- and (18*R*,19*R*)-**15** in MeOH (σ = 0.35 eV, shift = –2 nm for both isomers).

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The cytotoxicity of compounds **1**, **2**, **5**, and **7**–**26** was evaluated against three cancer cell lines, human hepatocellular carcinoma (HepG2), human gastric carcinoma (NCI-N87), and human breast carcinoma (MCF-7), by MTT assay[33] using doxorubicin as a positive control. As shown in Table 5, the new compound **2** exhibited growth inhibitory effects on all tested cancer cell lines with IC_{50} values ranging from 3.2 to 6.5 μM and compound **5** displayed the activity against HepG2 and NCI-N87 cell lines with the values around 5.0 μM. The known compound **14** gave the best activity against the tested cells with IC_{50} values being 1.9–4.3 μM, which was in agreement with the previous finding.[16] A preliminary structure-activity relationship (SAR) analysis revealed that the structural types and number of oxygenated functional groups in these triterpenoids influenced their cytotoxicity. In general, ursolic acid derivatives were more favorable to cytotoxicity than their oleanolic acid counterparts (**22**, **23** vs. **16**, **17**), and the increase of hydroxy groups on rings A and C and 20(30)-dehydrogenation in ursolic acid derivatives diminished the activity (**22**, **23** vs. **24**, **25**; **23** vs. **20**). For taraxast-20-en-28-oic acid derivatives, the substituent attached to C-14 played an important role in their cytotoxic activity. The 14-methyl (**14**) was slightly superior to the 14-carboxylic acid (**2**) while the 14-formyl (**1**) lost the activity (Table 5). Among the dicarboxylic acid 28-methyl esters, both olean-13(18)-ene **5** and taraxast-20-ene **2** exhibited good cancer cell growth inhibitory effects while the urs-20-ene **10** was non-cytotoxic.

Table 5. Cytotoxic Activity (IC_{50}, μM)[a] of Compounds 1, 2, 5, and 7–26

compound[b]	Cell lines		
	HepG2	NCI-N87	MCF-7
2	3.2 ± 0.49	6.5 ± 1.33	4.9 ± 0.52
5	5.0 ± 0.78	5.1 ± 0.84	> 10.0
14	1.9 ± 0.30	2.4 ± 0.02	4.3 ± 0.01
15	> 10.0	7.3 ± 0.28	> 10.0

22	> 10.0	4.2 ± 0.09	6.6 ± 0.32
23	> 10.0	4.4 ± 0.68	> 10.0
26	> 10.0	9.1 ± 0.56	> 10.0
doxorubicin	1.2 ± 0.02	1.6 ± 0.21	1.0 ± 0.17

[a]Values represent means ± SD based on three individual experiments. [b]Compounds **1**, **7**–**13**, **16**–**21**, **24** and **25** were non-cytotoxic (IC_{50} > 10.0 μM) against all tested cell lines.

The in vitro anti-inflammatory activity of compounds **1**, **2**, **7**–**13**, **15**, **17**–**21**, and **23**–**25** were assessed using previously described method.[34] As listed in Table 6, the 11-oxygenated oleanolic acid derivatives **7** and **8** exhibited inhibitory effects on LPS-induced IL-6 and TNF-α production in RAW 264.7 macrophages, of which, **7** inhibited the production of IL-6 by 68.1% and TNF-α by 53.7% at the minimal tested concentration 2 μM, which were comparable to those of the control drug dexamethasone at the same concentration. However, none of their ursolic acid counterparts (**11**, **12**, and **25**) provided inhibition more than 50% against any of the two cytokins at the tested concentrations. The major component cleisocalyxic acid B (**2**) displayed moderate inhibitory effects (51.0% and 42.9% inhibition for IL-6 and TNF-α, respectively) at the highest non-cytotoxic concentration (2 μM). Examination of inhibition of IL-6 and TNF-α production by the tested triterpenoids suggested that the 11α-hydroxylated oleanolic acid derivative **7** was the most favorable molecule for the activity, the methylation of OH-11 slightly weakened the activity (**7** vs. **8**), and the change of oxymethine at C-11 into a ketone group greatly diminished the activity (**7**, **8** vs. **9**).

Table 6. Inhibitory Effects of Compounds 1, 2, 7–13, 15, 17–21, and 23–25 on LPS-induced IL-6 and TNF-α Production in RAW 264.7 Macrophages (% Inhibition)[a, b]

Compound	IL-6				TNF-α			
	50 μM	10 μM	2 μM	0.4 μM	50 μM	10 μM	2 μM	0.4 μM

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1	nt[c]	53.8 ± 0.7	19.2 ± 5.3	23.6 ± 5.1	nt	35.9 ± 5.2	14.1 ± 2.0	14.8 ± 0.3
2	nt	nt	51.0 ± 0.2	38.4 ± 8.6	nt	nt	42.9 ± 2.1	39.1 ± 1.1
7	74.9 ± 2.0	69.9 ± 0.3	68.1 ± 0.2	nt	65.6 ± 0.3	57.7 ± 0.1	53.7 ± 0.3	nt
8	68.8 ± 1.5	64.8 ± 0.4	42.3 ± 2.9	nt	52.8 ± 1.1	54.1 ± 0.7	22.0 ± 1.4	nt
9	63.0 ± 0.1	35.2 ± 0.8	33.8 ± 0.5	nt	18.8 ± 1.6	< 10.0	< 10.0	nt
dexamethasone	84.9 ± 2.1	80.4 ± 0.2	75.9 ± 1.9	nt	86.1 ± 0.1	79.2 ± 0.8	71.8 ± 0.6	nt

[a]Values represent means ± SD based on three individual experiments. [b]Compounds **10**–**13**, **15**, **17**–**21**, and **23**–**25**, giving inhibition no more than 50% against any of the two cytokines at all tested concentrations, are not listed. [c]nt: not tested.

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EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained on a Perkin-Elmer 341 polarimeter with MeOH or $CHCl_3$ as solvent. ECD spectra of **3**, **6**, **9**, **13**, **15** in MeOH were recorded on a Chirascan CD spectrometer (Applied Photophysics Ltd., Leatherhead, Kent, UK) using a 50 nm/min scanning speed, 1 nm bandwidth, and three accumulations. The ECD spectrum of solvent MeOH was used as baseline and subtracted from the experimental spectrum. ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra were recorded in pyridine-d_5 or methanol-d_4 on a Brucker AVIII 500M instrument for ^{1}H at 500 and for ^{13}C at 125 MHz using the residual solvent peak as reference. Chemical shifts are reported as ppm (δ units) and the coupling constants as *J* in hertz (Hz). The splitting pattern abbreviations are as follows: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, brs = broad singlet. High-resolution electrospray ionization mass spectrometry (HRESIMS) was obtained on Bruker maXis Q-TOF mass spectrometer. ESIMS data were collected on a MDS Sciex API2000 LC/MS/MS instrument. Preparative HPLC was performed on a Waters 600 pump and a 2487 dual λ absorbance detector. All separations were carried out with a Shimadzu Shim-Pack Pro-ODS column (20mm × 25 cm)

at a flow rate of 5 mL/min. For column chromatography, silica gel 60 (100–200 mesh, Qingdao Marine Chemical Ltd., Qingdao, People's Republic of China), YMC ODS-A (75 μm, YMC Co. Ltd., Kyoto, Japan), and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) were used. Fractions were monitored by TLC and spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. Theoretical computations of ECD spectra were performed using the truncated structures of compounds (see Supporting Information) in order to reduce the computational cost.

Plant Material. The leaves of *Cleistocalyx operculatus* were collected from the South China Botanical Garden, Guangzhou, People's Republic of China, in March 2013 and authenticated by Professor Fuwu Xing. A voucher specimen (IBSC-0241915) was deposited at the South China Botanical Garden Herbarium (IBSC).

Extraction and Isolation. The fresh leaves of *Cleistocalyx operculatus* were dried at room temperature and then crushed into powder (3.3 kg). After extraction with 90% EtOH for 24 h (×3), the solutions were combined and concentrated under a vacuum. The crude extract was suspended in water and extracted successively with petroleum ether, EtOAc, and *n*-BuOH for three times each. The combined petroleum ether- and EtOAc-soluble fractions (350 g) were subjected to silica gel column chromatography (CC), eluted with gradient $CHCl_3$-MeOH mixtures (100:0–50:50), to afford ten fractions (Frs. A–J).

Fr. D (40 g) was further applied to silica gel CC using gradient petroleum ether-acetone mixtures (v/v, 9:1–6:4), to yield ten subfractions (Fr. D1–D10). Fr. D4 and D6 were separately subjected to Sephadex LH-20 CC using $CHCl_3$-MeOH (1:1) as an eluent and the corresponding subfractions were purified by preparative HPLC using 80% CH_3CN to yield **27** (30 mg) and **14** (40 mg) and using 90% CH_3CN to yield **1** (3 mg) and **3** (3 mg), respectively. The precipitates in

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Fr. D5, D8, and D9 in MeOH were collected by filtration and washed with MeOH to give **16** (30 mg), **2** (2.2 g), and **15** (50 mg), respectively. The filtrate of Fr. D8 was further separated by ODS CC using aqueous MeOH of decreasing polarities (50–100%), followed by preparative HPLC using 85% CH_3CN to yield **4** (3 mg) and using 90% CH_3CN to yield **5** (5 mg) and **22** (50 mg). Fr. E (35 g) was subjected to silica gel CC using petroleum ether-acetone mixtures of increasing polarities (9:1–2:1) to give nine subfractions (Fr. E1–E9). Fr. E4 was further separated by ODS CC using aqueous MeOH of decreasing polarities (50–100%), followed by preparative HPLC using 85% MeOH, to afford **28** (2 mg). The precipitate in Fr. E5 in MeOH was collected and washed with MeOH to yield a mixture of **17** and **23** (8 g), of which an aliquot (200 mg) was applied to preparative HPLC using 85% MeOH to give **17** (45 mg) and **23** (50 mg) in pure form. Fr. E6 was separated by ODS CC using aqueous MeOH (50–100%). The fraction obtained by elution of 80% MeOH was purified by preparative HPLC eluting with 80% MeOH to give **20** (6 mg). In turn, the fraction afforded by elution of 90% MeOH was purified with the same method using 85% MeOH to afford **10** (5 mg) and **26** (4 mg). Fr. G (18 g) was fractionated by ODS CC using aqueous MeOH (40–100%) to afford seven subfractions (Fr. G1–G7). Fr. G5 and G6 were chromatographed separately on a Sephadex LH-20 column using $CHCl_3$/MeOH (1:1) as an eluent and the resultant fractions were purified by preparative HPLC, using 65% and 70% MeOH, successively, to yield **6** (1 mg), **7** (9 mg), **8** (20 mg), **9** (15 mg), **11** (3 mg), **12** (3 mg), **13** (5 mg), **19** (22 mg), and **25** (4 mg). Fr. G7 in MeOH yielded a precipitate (10 g) that was collected by filtration and a portion (300 mg) was purified by preparative HPLC using 75% MeOH to give **18** (60 mg), **21** (20 mg), and **24** (65 mg).

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*Cleistocalyxic Acid A (**1**)*: white powder; $[\alpha]^{20}_{D}$ +9.1 (*c* 0.17, $CHCl_3$); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; (–)-ESIMS *m/z* 469 $[M - H]^-$; (–)-HRESIMS *m/z* 469.3323 $[M - H]^-$ (calcd for $C_{30}H_{45}O_4$, 469.3314).

*Cleistocalyxic Acid B (**2**)*: white powder; $[\alpha]^{20}_{D}$ +21.1 (*c* 0.2, $CHCl_3$); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 501 $[M + H]^+$, 523 $[M + Na]^+$; (–)-ESIMS *m/z* 499 $[M - H]^-$; (+)-HRESIMS *m/z* 501.3575 $[M + H]^+$ (calcd for $C_{31}H_{49}O_5$, 501.3575).

*Cleistocalyxolide A (**3**)*: white powder; $[\alpha]^{2}_{0}$ +16.4 (*c* 0.18, $CHCl_3$); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 471 $[M + H]^+$; (+)-HRESIMS *m/z* 963.6680 $[2M + Na]^+$ (calcd for $C_{60}H_{92}NaO_8$, 963.6684).

*Cleistocalyxic Acid C (**4**)*: white powder; $[\alpha]^{2}_{0}$ –1.1 (*c* 0.26, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 495 $[M + Na]^+$; (–)-ESIMS *m/z* 471 $[M - H]^-$; (+)-HRESIMS *m/z* 495.3442 $[M + Na]^+$ (calcd for $C_{30}H_{48}NaO_4$, 495.3445).

*Cleistocalyxic Acid D (**5**)*: white powder; $[\alpha]^{2}_{0}$ –27.0 (*c* 0.38, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 523 $[M + Na]^+$; (–)-ESIMS *m/z* 499 $[M - H]^-$; (–)-HRESIMS *m/z* 499.3445 $[M - H]^-$ (calcd for $C_{31}H_{47}O_5$, 499.3429)

*Cleistocalyxolide B (**6**)*: white powder; $[\alpha]^{2}_{0}$ +53.0 (*c* 0.1, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 1 and 2; (+)-ESIMS *m/z* 527 $[M + Na]^+$; (–)-ESIMS *m/z* 503 $[M - H]^-$, 539 $[M + Cl]^-$; (–)-HRESIMS *m/z* 539.3151 $[M + Cl]^-$ (calcd for $C_{30}H_{48}ClO_6$, 539.3145)

*Cleistocalyxic Acid E (**7**)*: white powder; $[\alpha]^{2}_{0}$ +18.9 (*c* 0.8, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 527 $[M + Na]^+$; (+)-HRESIMS *m/z* 527.3325 $[M + Na]^+$ (calcd for $C_{30}H_{48}NaO_6$, 527.3343).

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Cleistocalyxic Acid F ***(8)***: white powder; $[\alpha]^{2}_{0}$ +14.3 (*c* 2.3, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 541 $[M + Na]^+$, 557 $[M + K]^+$; (–)-ESIMS *m/z* 517 $[M – H]^–$; (+)-HRESIMS *m/z* 541.3506 $[M + Na]^+$ (calcd for $C_{31}H_{50}NaO_6$, 541.3500)

Cleistocalyxic Acid G ***(9)***: white powder; $[\alpha]^{2}_{0}$ +63.6 (*c* 0.3, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 525 $[M + Na]^+$, 541 $[M + K]^+$; (–)-ESIMS *m/z* 501 $[M – H]^–$; (–)-HRESIMS *m/z* 501.3227 $[M – H]^–$ (calcd for $C_{30}H_{45}O_6$, 501.3222).

Cleistocalyxic Acid H ***(10)***: white powder; $[\alpha]^{2}_{0}$ +39.9 (*c* 0.29, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 539 $[M + Na]^+$; (–)-ESIMS *m/z* 515 $[M – H]^–$; (–)-HRESIMS *m/z* 515.3383 $[M – H]^–$ (calcd for $C_{31}H_{47}O_6$, 515.3378).

Cleistocalyxic Acid I ***(11)***: white powder; $[\alpha]^{2}_{0}$ +45.3 (*c* 0.3, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 525 $[M + Na]^+$; (+)-HRESIMS *m/z* 525.3178 $[M + Na]^+$ (calcd for $C_{30}H_{46}NaO_6$, 525.3187).

Cleistocalyxic Acid J ***(12)***: white powder; $[\alpha]^{2}_{0}$ +49.5 (*c* 0.15, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 539 $[M + Na]^+$; (–)-ESIMS *m/z* 515 $[M – H]^–$; (–)-HRESIMS *m/z* 515.3387 $[M – H]^–$ (calcd for $C_{31}H_{47}O_6$, 515.3378).

Cleistocalyxic Acid K ***(13)***: white powder; $[\alpha]^{2}_{0}$ +110.0 (*c* 0.1, CH_3OH); ^{1}H and ^{13}C NMR data, see Tables 3 and 4; (+)-ESIMS *m/z* 523 $[M + Na]^+$, 539 $[M + K]^+$; (–)-ESIMS *m/z* 499 $[M – H]^–$; (–)-HRESIMS *m/z* 499.3070 $[M – H]^–$ (calcd for $C_{30}H_{43}O_6$, 499.3065).

3β,21α-Dihydroxytaraxast-28,20β-olide ***(15)***: white powder; $[\alpha]^{2}_{0}$ –1.1 (*c* 0.17, CH_3OH); ^{1}H NMR (500 MHz, pyridine-d_5) δ 4.14 (1H, dd, *J* = 10.2, 5.7 Hz, H-21), 3.49 (1H, dd, *J* = 10.2, 6.1 Hz, H-3), 2.35 (1H, ddd, *J* = 14.0, 13.4, 4.2 Hz, H-15*β*), 2.25 (1H, dd, *J* = 13.3, 10.2 Hz, H-22*β*), 2.06 (1H, ddd, *J* = 13.7, 4.2, 2.6 Hz, H-16*β*), 1.90 (2H, m, H-2), 1.87 (1H, m, H-22*α*), 1.82 (1H,

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m, H-19), 1.70 (1H, ddd, *J* = 13.0, 4.4, 3.3, H-1*β*), 1.66 (3H, s, H-30), 1.65 (1H, m, H-12*β*), 1.56 (1H, m, H-6*α*), 1.54 (1H, m, H-11*α*), 1.49 (1H, m, H-18), 1.46 (3H, d, *J* = 7.3 Hz, H-29), 1.39 (2H, m, H-7), 1.38 (1H, m, H-6*β*), 1.37 (2H, m, H-9 and H-13), 1.36 (1H, m, H-16*α*), 1.26 (3H, s, H-23), 1.19 (1H, m, H-11*β*), 1.14 (1H, ddd, *J* = 14.0, 4.7, 2.6, H-15*α*), 1.05 (3H, s, H-24), 0.99 (1H, m, H-1*α*), 0.97 (6H, s, H-26 and H-27), 0.86 (3H, s, H-25), 0.82 (1H, m, H-5); ^{13}C NMR (125 MHz, pyridine-d_5) δ 176.9 (C-28), 85.4 (C-20), 78.6 (C-3), 72.1 (C-21), 56.4 (C-5), 51.3 (C-9), 49.5 (C-18), 43.8 (C-13), 43.4 (C-22), 43.1 (C-19), 42.7 (C-17), 41.9 (C-14), 41.3 (C-8), 40.0 (C-4), 39.8 (C-1), 37.9 (C-10), 34.9 (C-7), 29.2 (C-23), 28.8 (C-2), 28.5 (C-16), 28.0 (C-15), 25.9 (C-12), 22.4 (C-30), 21.7 (C-11), 19.5 (C-29), 19.2 (C-6), 17.1 (C-25), 16.8 (C-24), 16.4 (C-26), 14.9 (C-27); (+)-ESIMS *m/z* 473 $[M + H]^+$, 495 $[M + Na]^+$; (+)-HRESIMS *m/z* 495.3441 $[M + Na]^+$ (calcd for $C_{30}H_{48}NaO_4$, 495.3445).

Cytotoxicity Assay. Human hepatocellular carcinoma (HepG2), human gastric carcinoma (NCI-N87), human breast carcinoma (MCF-7), and murine macrophage RAW 264.7 cell lines were purchased from Kunming Institute of Zoology, Chinese Academy of Sciences (Kunming, People's Republic of China). The cells were cultured in RPMI 1640 (for HepG2 and NCI-N87 cells) and DMEM (for MCF-7 and RAW 264.7 cells) medium supplemented with 10% heated-inactivated fetal bovine serum (Gibco, Carlsbad, CA, USA) as well as 1% penicillin and streptomycin in a 37 °C, 5% CO_2 incubator. Cell viability was assessed using the MTT method as previously described.[33] All experiments were performed in triplicate, and the data are expressed as means ± SD of three independent experiments. Doxorubicin was used as positive control.

Anti-Inflammatory Activity Assay. The murine macrophage RAW 264.7 cells were cultured in DMEM medium supplemented with 10% heated-inactivated fetal bovine serum (Gibco) as

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well as 1% penicillin and streptomycin in a 37 °C, 5% CO_2 incubator. The working concentrations of test compounds were set as 50, 10, 2 μM for **7**–**9**, **12**, **13**, and **19**, 10, 2, 0.4 μM for **1**, **10**, **11**, **15**, **17**, **18**, **20**, **21**, and **23**–**25**, and 2, 0.4, 0.08 μM for **2**, according to the MTT assay results, in which test compounds at the highest serial concentration were non-cytotoxic toward RAW 264.7 cells (growth inhibition less than 5%). Anti-inflammatory activity was evaluated using previously reported method[34] with minor modifications. In brief, macrophage RAW 264.7 cells were pretreated with serial concentrations of test triterpenoids at 37 °C for 1 h, followed by the stimulation with LPS (0.1 μg/ml) for an additional 24 h. Cell supernatants were evaluated for the production levels of IL-6 and TNF-α using commercial enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN, USA). Dexamethasone was used as positive control. All values were given as means ± SD (n = 3).

ASSOCIATED CONTENT

Supporting Information. The 1D, 2D NMR and HR-ESIMS spectra of compounds **1**–**13** and **15**, and ~~for~~ theoretical computations of ECD spectra are available free of charge via the Internet at http://pubs.acs.org.

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Table of Contents Graphic

Bioactive Pentacyclic Triterpenoids from the Leaves of *Cleistocalyx operculatus*

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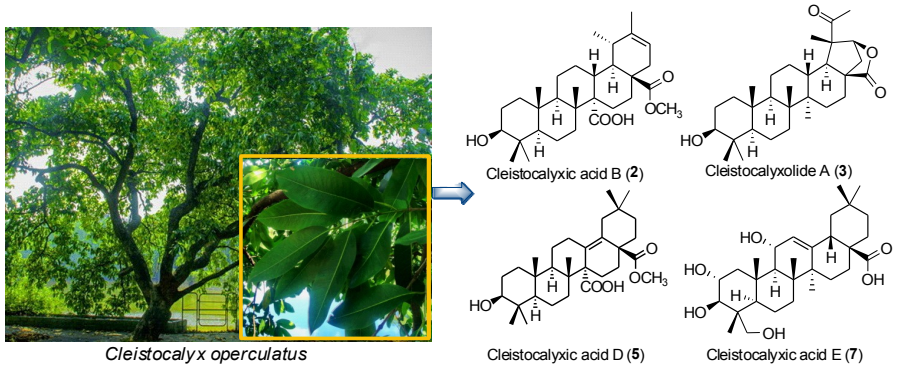

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